ENVIRONMENTS THAT MAINTAIN FUNCTION OF PRIMARY LIVER CELLS

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates generally to useful surfaces for culturing primary liver cells *in vitro*, and to methods using those surfaces.

Description of the Background Art

Typically, for cell culture, cells are dispersed in a culture medium supplemented with serum, and the culture medium is then dispensed into a vessel that is made of a synthetic cell culture substrate such as tissue culture-grade polystyrene (PS). Under these conditions, non-specific protein adsorption to the PS surface rapidly occurs, generating a protein layer comprised of many different serum proteins in a spectrum of conformational states ranging from almost native to highly denatured. In stationary cultures, the cells subsequently settle to the surface and start to "interrogate" this poorly organized interface via cellular integrins, proteoglycans and selectins on their surface. Interactions with this randomly adsorbed protein layer lead to arbitrary biological responses that affect a variety of processes, including cell attachment (or adherence), spreading, proliferation, migration and differentiation. By contrast, *in vivo*, normal biological reactions occur via specific and organized ligand-receptor interactions, which in turn trigger highly organized signaling processes.

Thus, there is a need for highly defined cell culture surfaces that mimic the *in vivo* specificity of biological events to more effectively support desired cell biological activities during *in vitro* culture.

The sera conventionally used for cell culture, which includes undefined mixtures of proteins that vary from lot to lot of serum, can create further unwanted complications. For example, when cells are being prepared for *in vivo* uses such as cell therapy in humans, prior use of serum in culture can introduce into the cell preparation (1)

biohazardous substances and (2) animal products that can induce unwanted immune responses in recipients.

Thus, there is a need for cell culture methods that employ serum-free, chemically defined, culture media that provide the same benefits during culture as do sera. There is a further need for serum-free cell culture and methods thereof for primary liver cells, many of which lose some of their natural function when cultured *in vitro*. For example, primary hepatocytes lose the ability to produce the protein albumin, a function of healthy cells.

The present invention is intended to meet the above needs by providing highly defined cell culture surfaces, which comprise, *inter alia,* extracellular matrix (ECM) proteins and active factors. Among the advantages of these new surfaces is that they enable the reduction of serum concentrations or the complete avoidance of serum *in vitro*.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide compositions and methods suitable for the culture of mammalian cells, in particularly primary liver cells. Preferred cells for use in the invention are liver cells such as primary hepatocytes.

In one aspect, the present invention provides a surface particularly suited for use in cell culture comprising a cell adhesion resistant (CAR) material and, bound to the CAR material, one or more ECM proteins or a biologically active fragment or variant thereof and, optionally, one or more active factors or a biologically active fragment or variant thereof. By "biologically active" is meant that the fragment or variant has essentially the same activity in promoting cell attachment and maintaining function as does the full-length unmodified ECM protein or active factor. Cell "attachment" means binding of the cell to the surface such that the cell is not eluted by conventional washing or handling procedures. By "maintaining function" is meant that the cells produce albumin or maintain cytochrome P450 activity.

By "ECM protein" is meant an extracellular matrix protein that can be used to mediate cell attachment and growth. (For more description of ECM proteins, see E.D. Hay, ed., *Cell Biology of Extracellular* Matrix, 2nd ed., Plenum Press, New York, 1991.)

Examples of ECM proteins in this method include elastin, fibronectin, vitronectin, laminin, and a collagen, such as collagen I, collagen III, collagen IV, or collagen VI.. Particularly preferred are elastin, collagen I, collagen IV and collagen VI. Most particularly preferred are collagen I and collagen IV.

In preferred embodiments, the active factor is a naturally- or non-naturally-occurring polycationic polymer, or a biologically active fragment or variant thereof, that promotes cell attachment, survival or function when presented to the cells along with the ECM protein. Polycationic polymers, such as polyethyleneimine (PEI), poly-D-lysine (PDL), poly-L-lysine (PLL), poly-D-ornithine (PDO) or poly-L-ornithine (PLO), may be used. In particularly preferred embodiments, the active factor is poly-L-lysine and poly-D-ornithine.

The present inventors found, surprisingly, that the present surfaces promote the attachment and maintenance of function of primary liver cells as well as, and often better than, standard culture surfaces using conventional conditions (*e.g.*, incubation on conventional tissue culture polystyrene using commercial culture media, either with or without serum). Additionally, certain combinations of ECM proteins and/or active factors (ECM protein compositions) promoted cell attachment and function more so than other combinations. These improved effects are preferably achieved using chemically defined, serum-free media.

Advantages of this invention include:

1) The use of defined mammalian cell culture conditions, which allows the cell attachment process to be controlled by the ECM protein(s) bound to the cell culture substrate, rather than by nonspecifically (randomly and arbitrarily) adsorbed serum proteins forming a layer on the culture substrate and eliminates the need to use other uncharacterized or unpurified animal products, such as Matrigel™;

2) The ability to attribute specific cellular processes to specific ECMs, which eliminates the intermixed biological effects of ECM proteins with those other biological factors present in conventional serum-supplemented culture media;

3) The use of covalently bound ECMs and/or active factors attached to the surface (rather than being passively adsorbed), which restricts the ECMs and/or active

factors to the substrate and prevents desorption into the liquid phase (culture medium) and also increases cell attachment by preventing solubilized ECMs and/or active factors on passive coatings from blocking attachment sites on suspended cells; and

4) The ability to gain faster regulatory approval because serum is significantly reduced or eliminated, which eliminates or significantly reduces biohazardous agents, immunogenic or otherwise harmful products.

One aspect of the invention is a surface comprising (a) a cell adhesion resistant (or resistive) (CAR) material, and (b) bound to the CAR material, one or more ECM proteins or a biologically active fragment or variant thereof, and, optionally, one or more active factors, or a biologically active fragment or variant thereof. Examples of ECM proteins are elastin, fibronectin, vitronectin, laminin, or a collagen, such as collagen I, collagen III, collagen IV or collagen VI. Particularly preferred are collagen I, collagen IV and collagen VI.

As used herein, the term "CAR material" refers to a material that, when present on a surface, prevents, inhibits, or reduces the non-specific binding (adhesion) to the support of cells or proteins or polypeptides found on cell surfaces. CAR materials and surfaces are resistant to mammalian cells and preferably also to microorganisms. CAR materials and surfaces are sometimes referred to as "non-fouling substrates," "inert coatings," "low affinity reagents," or "non-adhesive coatings". Examples of CAR materials include hyaluronic acid (HA) or a derivative thereof, alginic acid (AA) or a derivative thereof, polyhydroxyethylmethylacrylate (poly-HEMA), polyethylene glycol (PEG), glyme or a derivative thereof, polypropylacrylamide, polyisopropylacrylamide, or a combination of these compounds. Preferably, the CAR material is HA.

In some embodiments, one or more of a proteoglycan, a biglycan, a glycosaminoglycan, or Matrigel™ may be bound to the CAR material.

The ECM proteins and active factors may be bound either covalently or non-covalently to the CAR surface, but are preferably bound covalently.

In one embodiment, the CAR material is attached to the support by treating the support with an oxidizing plasma, and binding the CAR material to the treated support. In another embodiment, the CAR material is attached to the support by treating the

support with an oxidizing plasma; exposing the treated support to a polycationic polymer with amino groups to form an intermediate layer; and binding the CAR material to the intermediate layer. Preferably, the polycationic polymer is polyethylene imine (PEI) or poly-L-lysine (PLL). (See for example, U.S. Patent 6,129,956 be Morra et al.)

The support may be a natural or synthetic organic polymer, or an inorganic composite. Suitable supports include polystyrene (PS), polypropylene, polyethylene, polyethylene terephthalate, polytetrafluoroethylene, polylactide, cellulose, glass, or ceramic. Preferably, the support is PS.

The invention is also directed to a cell culture comprising a surface of the invention as described above. The culture may be grown in a cell culture vessel, such as a slide, a multi-well plate, a culture dish, a culture flask, a culture bottle, *etc.* The culture may also be grown on a flexible substrate or a 3-dimensional (3D) scaffold.

Another aspect of the invention is a method for promoting the attachment and maintenance of function of primary liver cells in culture. The method comprises contacting the cell in a culture medium with a surface of the invention under conditions effective for the attachment and maintenance of function of the cell. Examples of surfaces are those comprising (a) a support to which is bound a CAR material, and (b) one or more ECM proteins (or a biologically active fragment or variant thereof). Examples of ECM proteins in this method include elastin, fibronectin, vitronectin, laminin, and a collagen, such as collagen I, collagen III, collagen IV and collagen VI. Also, optionally bound to the CAR surface is (c) one or more active factors, for example, a polycationic polymer such as, as polyethyleneimine (PEI), poly-D-lysine (PDL), poly-L-lysine (PLL), poly-D-ornithine (PDO) or poly-L-ornithine (PLO). The addition of the active factor bound to the CAR surface creates an ECM protein composition attached to the CAR surface.

Another aspect of the invention is a method for identifying a test agent that stimulates or inhibits attachment or function of primary liver cells in culture, comprising (a) contacting the cells in a serum-free culture medium with a surface of the invention plus the test agent; and (b) measuring the attachment and function of these cells compared to attachment and function of control cells without the test sample. Increased

attachment or function in the presence of the test agent indicates the presence of a factor that stimulates cell attachment or function, and decreased attachment and function in the presence of the test agent indicates the presence of a factor that inhibits cell attachment and function. This method may be used to identify a potential drug target, to determine the effect of an agent on a property of the cell, or to determine if a potential agent is toxic to the cell, *etc.*

Liver cells cultured according to the present invention may be contained in or on a device or scaffold suitable for cell therapy, as will be evident to persons of skill in the art.

The embodiments described above and throughout the specification are particularly preferred for use with primary liver cells. Liver cell types that may be used include primary hepatocytes from any species. Rat and human primary hepatocytes are described herein.

In the embodiments of the present invention, the culture medium may be supplemented with serum, but is preferably serum-free. A suitable, defined serum-free medium, BD Hepato-STIM™ medium, is described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows attachment and maintenance of cell function (CYP activity) of human primary hepatocytes .

Figure 2 shows attachment and maintenance of cell function (CYP activity) of rat primary hepatocytes.

Figure 3 shows attachment and maintenance of albumin secretion of human primary hepatocytes.

Figure 4 shows attachment and maintenance of albumin secretion of rat primary hepatocytes.

Figure 5 shows morphology of primary hepatocytes.

DETAILED DESCRIPTION OF THE INVENTION

Surfaces of the invention comprise a solid, preferably polymeric, support having CAR properties. The support may take any of a variety of forms. It may be of any

suitable shape, such as square, rectangular, circular or polygonal, and can be two- or three-dimensional. It may be any of a variety materials, including natural polymers, synthetic polymers and inorganic composites. Natural polymers include, *e.g.*, collagen and glycosaminoglycan (GAG)-based materials. Synthetic polymers include, *e.g.*, poly(a-hydroxy acids) such as polylactic acid (PLA), polyglycolic acid (PGA) and copolymers thereof (PLGA), poly(ortho ester), polyurethanes, and hydrogels, such as polyhydroxyethylmethacrylate (poly- HEMA) or polyethylene oxide-polypropylene oxide copolymer. Hybrid materials, containing naturally derived and synthetic polymer materials, may also be used; non-limiting examples of such materials are disclosed in Chen *et al.* (2000), *Advanced Materials 12*:455-457. Inorganic composites include, *e.g.*, calcium phosphate ceramics, bioglasses and bioactive glass-ceramics, in particular composites combining calcium hydroxyapatite and silicon stabilized tricalcium phosphate. Among preferred supports are polystyrene (PS), polypropylene, polyethylene, polyethylene terephthalate, polytri- or tetra-fluoroethylene, polyhexafluoropropylene, polyvinyl chloride, polyvinylidine fluoride, polylactide, cellulose, glass, or a ceramic. In a preferred embodiment, the support is part of a tissue culture vessel, such as a PS tissue culture dish or multi-well plate.

Alternatively, the surface may be treated, for example, using plasma treatments known in the art and described in U.S. Application 10/259,797. Any suitable CAR material, many of which are known to those skilled in the art, may be bound to the support. Typical CAR materials include hyaluronic acid (HA) or a derivative thereof, alginic acid (AA) or a derivative thereof, poly-HEMA, polyethylene glycol (PEG), glyme or a derivative thereof, polypropylacrylamide, and polyisopropylacrylamide. Combinations of CAR materials may also be used. In a preferred embodiment, the CAR material is HA.

The CAR material is preferably bound to the support by covalent bonds. Various types of covalent bonds can form, some of which are discussed in more detail in co-pending, commonly assigned U.S. patent applications, all hereby incorporated by reference: U.S. Patent Application Serial Number 10/259,797 by Andrea Liebmann-Vinson and R. Clark, filed September 30, 2002; U.S. Patent Application Serial Number

10/260,737 by Mohammad A. Heidaran and Mary K. Meyer entitled Method and Apparatuses for the Integrated Discovery of Cell Culture Environments, filed September 30, 2003; U.S. Patent Application Serial Number 10/259,815 by John J. Hemperly, entitled Proliferation and Differentiation of Stem Cell from Bone Marrow and Other Cells Using Extracellular Matrix and other Molecules, filed September 30, 2002; attorney docket number 7767-184045, filed August 15, 2003, and attorney docket number 7767-183015, filed September 12, 2003. These applications also disclose other aspects of making and using surfaces that include supports with bound CAR materials and ECM proteins.

In one embodiment, one or more ECM proteins (or a biologically active fragment or variant thereof) and, optionally, one or more active factors (a biologically active fragment or variant thereof) are bound to the CAR material. Preferred embodiments speak to the following combinations: collagen I + poly-L-ornithine; and collagen IV + poly-L-ornithine; and collagen VI and elastin.

The ECM protein(s) can be in the form of a naturally occurring polypeptide (protein), a recombinant polypeptide, or a synthetic or semi-synthetic polypeptide, or any combination thereof. The terms "polypeptide" and "protein" are used interchangeably herein.

Methods of cloning, expressing and purifying polypeptides, such as ECM proteins, are conventional, as are methods of generating synthetic or semi-synthetic polypeptides. ECM proteins can also be obtained from commercial sources.

Biologically active fragments or variants of other ECM proteins and active factors can also be bound to the CAR material. As used herein, the term "a biologically active fragment or variant" includes a polypeptide that retains substantially at least one of the biological functions or activities of the wild type polypeptide. For example, a biologically active fragment or variant (of an ECM protein) is one that can bind to a CAR material, while retaining the ability to promote the attachment and function of a cell when used in a method of this invention.

Preferred ECM proteins for binding to a CAR surface and use herein include elastin, collagen I, collagen IV, and collagen VI. Preferred active factors include poly-D-lysine and poly-L-ornithine.

The ECM proteins and active factors can be bound to the CAR material either covalently or non-covalently (*e.g.*, passively adsorbed, such as by electrostatic forces, ionic or hydrogen bonds, hydrophilic or hydrophobic interactions, Van der Waals forces, *etc.*). In a preferred embodiment, the binding is covalent. Co-pending U.S. patent applications 10/259,797, 10/260,737 and 10/259,815 describe such covalent binding of molecules to CAR surfaces.

Methods of making surfaces in which a CAR material is bound to a support, and in which ECM proteins are bound to the CAR material, are described in detail in co-pending U.S. patent applications 10/259,797, 10/260,737 and 10/259,815. In brief, one method of attaching a CAR material to a support comprises treating the support with an oxidizing plasma, and binding the CAR material to the treated support. Another method of attaching a CAR material to a support comprises treating the support with an oxidizing plasma; exposing the treated support to a polycationic polymer with amino groups (such as polyethyleneimine (PEI), poly-L-lysine (PLL), poly-D-lysine (PDL), poly-L-ornithine (PLO), poly-D-ornithine (PDO), poly(vinylamine) (PVA) or poly(allylamine) (PAA), preferably, PEI or PLL) to form an intermediate layer; and binding the CAR material to the intermediate layer. Methods of binding an ECM or a polycationic polymer to a CAR material are conventional. These include, *e.g.*, sodium periodate oxidation and reductive amination, *etc.*

In a particular embodiment of the invention, HA can be bound to PS to create the CAR surface using methods such as those described in Morra et al. (U.S. Pat. No. 6,129,956). Polystyrene culture dishes, 96-well plates or slides are exposed to an oxidizing radiofrequency plasma treatment, followed by exposure to a polyethyleneimine (PEI) solution to introduce reactive amine groups on the surface. A carbodiimide/succinimide supported condensation reaction of a primary amine with a carboxylic acid is used to form a covalent bond between the PEI coating and the

polysaccharide. Alternatively, amine groups introduced on polystyrene surfaces during the Primaria™ plasma treatment or on a polylysine coating (instead of PEI) can be used.

Next, conventional bioconjugation techniques including sodium periodate oxidation and reductive amination, are used to covalently couple the ECM protein to the inert HA. Any non-covalently attached extracellular matrix protein is removed by a salt-acid wash followed by rigorous rinsing with water. This process creates a well-defined surface consisting of covalently immobilized extracellular matrix protein on a non-fouling (=eliminating non-specific cell attachment) background provided by HA.

Alternatively, alginate (also known as alginic acid) can be used as the non-adhesive background and ECM proteins can be immobilized onto this surface using the same chemistry as described above for HA. Also, other commonly known non-adhesive surfaces, such as poly-HEMA or PEG (also known as PEO) could be used in combination with a variety of chemistries to couple ECM proteins that are described in the literature. (*See* Hubbell, J.A., Biomaterials in Tissue Engineering, *Biotechnology*, 1995. 13: p. 565-76.)

A variety of articles may comprise a surface of the invention. Suitable articles will be evident to those of skill in the art. Such articles include cell culture vessels, such as slides (*e.g.*, tissue slides, microscope slides, *etc.*), plates (*e.g.*, culture plates or multi-well plates, including microplates), flasks (*e.g.*, stationary or spinner flasks), bottles (*e.g.*, roller bottles), bioreactors, or the like.

In addition to the more traditional two-dimensional culture surfaces and vessels described above, the present invention includes the use of three-dimensional (3D) scaffolds for use in conjunction with the ECM protein compositions of the present invention (including for testing candidate peptides for CAP activity when they are on a CAR surface). "Three-dimensional scaffold" refers herein to a 3D porous template that may be used for initial cell attachment and subsequent tissue formation either *in vitro* or i*n vivo*. A 3D scaffold according to this invention comprises base materials such natural polymers, synthetic polymers, inorganic composites and combinations of these materials, a CAR layer and bound thereto ECM proteins, and optionally, active factors, which promote or enhance cell attachment and function. 3D scaffolds are discussed in further

detail in copending, commonly assigned U.S. Patent Application, docket no. 7767-184045, filed August 15, 2003, and U.S. application no. 10/259,817, filed September 30, 2002.

This invention also speaks to the use of flexible substrates in culture. For example, Flexercell culture systems from Flexcell International Corporation are able to apply tensile, compressive or shear stresses to cultured cells. (See, for example, U.S. Patent Nos. 4,789,601, 4,822,741, 4,839,280, 6,037,141, 6,048,723, and 6,218,178.) U.S. Pat. No. 6,057,150 discloses the application of a biaxial strain to an elastic membrane that may be coated with extracellular matrix proteins and covered with cultured cells. U.S. Pat. No. 6,107,081 discloses another system in which a unidirectional cell stretching device comprising an elastic strip is coated with an extracellular matrix on which cells are cultured and stretched. A flexible substrate can be deformed easily and in a controlled manner, and also supports cell adhesion and growth comparable to conventional cell culture substrates. Silicones, such as poly(dimethyl siloxane) (PDMS), are particularly suitable for this application because they are not only highly flexible but also provide optical clarity that allows microscopic observation of the cell cultures.

The invention relates to a method of promoting the attachment and function of a primary liver cell in culture, comprising contacting the cell in a culture medium with a surface of the invention.

The cell may be "contacted" or brought into contact with the surface by any suitable means. For example, cells in a culture medium may be poured, pipetted, dispensed, *etc.*, into a culture vessel comprising the surface, or a medical device or scaffold comprising the surface may be submerged in culture medium in which the cells are suspended.

Any of the inventive surfaces described herein are suitable for this method. In one embodiment, the surface comprises an ECM protein bound to HA and, optionally, an active factor attached the CAR surface. In a preferred embodiment, the support is PS; the CAR material is HA; the ECM protein(s) is/are one of more of elastin, fibronectin, vitronectin, collagen I, collagen III, collagen IV, and collagen VI; and the ECM proteins are covalently bound to the HA. In a further preferred embodiment, an active factor,

poly-L-ornithine or poly-D-lysine, is bound the CAR surface, creating an ECM protein composition covalently bound the HA. The Examples herein describe the use of some combinations of ECM proteins and active factors in the present methods. Of course, other combinations can also be used.

Any of a variety of culture media may be used in conjunction with the inventive surfaces in the present methods. Commercially available media, such as DMEM, F12, αMEM, Hepato-STIM™, RPMI, or combinations thereof, may be used, either in the presence or absence of serum. Suitable sera include calf serum, fetal calf serum, horse serum, or the like. Preferably, a synthetic, chemically-defined, serum-free medium is used. A variety of suitable chemically defined media will be evident to the skilled worker. One such medium, BD Hepato-STIM™ (BD Biosciences, BD Discovery Lab Ware) medium, is employed in the Examples.

In the above methods, a cell is contacted with a surface of the invention under conditions effective for the attachment and maintenance of function of the cell. By "effective" conditions is meant conditions that result in a measurable amount of cell attachment and maintenance of function. Effective conditions can be readily determined and/or optimized by a skilled worker, using conventional methods. Among the factors to be varied include, *e.g.*, the vessel, culture medium, temperature, O_2/CO_2 concentrations, and the like. Some typical effective conditions are described in the Examples.

Another aspect of the invention is a method for identifying a test agent that modulates (*e.g.*, stimulates, inhibits, potentiates, *etc.*) attachment of a cell in culture, comprising (a) contacting the cell, in a culture medium lacking serum, with a surface of the invention and with the test agent suspected of including the factor; and (b) measuring the attachment of the cell compared to attachment of a similar cell in a culture in the absence of the test agent, wherein (i) increased attachment in the presence of the test agent indicates the presence in the test sample of a factor that stimulates attachment of the cell, and (ii) decreased attachment in the presence of the test agent indicates the presence in the sample of a factor that inhibits attachment of the cell. The comparison can be made to a cell to which the test agent has not been added, which is grown in parallel with the test agent; or the comparison can be made to a reference database.

One of skill in the art will recognize a variety of types of agents that can be tested in this method. For example, the method can be used to test putative drugs (*e.g.*, proteins, peptides, small molecules, nucleic acids, such as antisense molecules, ribozymes or RNAi, or the like) that affect an activity of a cell of interest (*e.g.*, an intercellular signalling cascade, a metabolic pathway, *etc.*). In addition to drug screening, drug discovery, and the identification of potential drug targets, the method can be used to determine if a potential agent is toxic to the cell and has a measurable detrimental effect, induces unregulated proliferation (oncogenic transformation), *etc.*

In another embodiment, the agent tested is a putative factor that can induce, enhance, or maintain a marker of interest, or that is important for the maintenance of a desirable cellular function. Typically, such markers/functions that can be studied in liver cells include (1) the induction of drug/toxin metabolizing enzymes of the cytochrome P450 family (CYP), an important hepatocyte function; or (2) the production of albumin, a function that is usually lost during upon primary culture of hepatocytes.

Among the types of agents that can be tested are proliferation factors, such as angiopoietin 2, BMP2, BMP4, erythropoietin, aFGF, bFGF, HGF, insulin, noggin, PDGF, TNF, VEGF, stem cell factors, GDF6, CSF, FH3/F2, TGFβ, or the like. Alternatively, one can test small molecules generated by conventional combinatorial chemistry, or peptide libraries. (See, for example, copending U.S. patent applications 10/260,737 and 10/259,816). Other types of agents will be evident to the skilled worker.

Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLES

MATERIALS AND METHODS

Rat Primary Hepatocytes were purchased from XenoTech, LLC (Lenexa, KS) and were shipped within 3 hours of isolation to BD Technologies. Human primary hepatocytes were isolated by BD Gentest (Woburn, MA) and shipped within 12-15 hours

in commercial organ preservation media (ViaSpan™). Human or rat cells in suspension were isolated using standard collagenase digestion methods.

Cell were re-suspended in fully supplemented BD Hepato-STIM™ medium (cat # 355056) and seeded at an initial density of 20,000 cells/well in fully supplemented BD Hepato-STIM™ medium and were placed into plates with various combinations of extracellular matrix proteins covalently coupled to a non-fouling surface. Plates were placed in an incubator at 5% CO_2 and 37°C and were allowed to incubate for 4, 6, or 7 days. BD Hepato-STIM™ medium was changed every other day by removing half the volume of media from the plates and adding the same volume of fresh medium.

On either day 6 or 7, triplicate plates were taken for assays as described below. Three assays were run on the same plates: CYP 1A1/2A activity assay using 7-ethoxy resorufin, an albumin enzyme-linked immunosorbent assay (ELISA) for albumin secretion, and an assay for determining cell number (MTT, nuclear counting or picogreen assay). The experiments were repeated with two separate rat liver preparations and one human liver preparation.

CYP1A1 activity assay and cell enumeration with nuclear stains

All media were transferred to separate plates and media samples were frozen at -20°C until ELISA assays for albumin secretion could be performed (see below). 5 uM 7-ethoxyresorufin + 80 uM dicumerol was added to all wells with cells and read at intervals for 30 min on a BMG Polarstar at excitation= 540 nm and emission=590 nm to detect CYP1A1/1A2 activity.

Immediately after the CYP1A1 activity assay, the resorufin solution was removed and cell numbers were determined using a variety of the following methods.

1) Cell number by nuclear staining: 7- ethoxyresorufin was aspirated and nuclear stain with 10 uM Hoechst 33334 stain (Molecular probes, cat # 3570) and 2 mM ethidium homodimer-1 (Molecular probes, Dead stain cat # L-3324) in BDT base media was added to each well. Plates were incubated for 30 min at room temperature and fluorescence images were captured an HT Imager (Discovery-1, Universal Imaging Corporation, a subsidiary of Molecular Devices, Downington, PA) at excitation of 405

nm and emission of 480 nm for the Hoechst stain and excitation of 535 nm and emission of 750 nm for the ethidium homodimer stain (10X magnification, 4 sites per well). UIC Metamorph™ analysis software was used for counting cells. Number of live cells was determined by subtracting total cells by dead cells (Hoechst stain-dead stain). Data are presented as total signal at 30 minutes divided by the cell number. CYP Activity data are presented in Figure 1 and Figure 2.

2) Cell number by MTT assay was determined using CellTiter 96® Non-Radioactive Cell Proliferation Assay.

3) Cell number by picogreen DNA assay was determined using Picogreen™ DNA dsDNA Quantitation Kit from Molecular Probes (cat. # P7589).

Albumin ELISA assays:

To measure albumin secretion in media samples, Probind Assay plates (Falcon 353915) were coated with 2 ug/ml Sheep IGG Anti-rat albumin antibodies (unconjugated, Cappel cat # 55729) in a bicarbonate buffer (pH=9.6) and allowed to incubate overnight at 4°C. Antibody plates were washed 3 X with PBS Tween 20 and blocked with 1% gelatin (Type B, 75 bloom, Sigma cat #G6650) in PBS Tween 20 for 30 min at 37°C. Blocking solution was rinsed off 3X with PBS Tween 20 and 1:400 diluted albumin (media samples) from ECM test plates. Plates were incubated 1 hr at 37°C, washed 3X with PBS Tween 20, and conjugated anti-albumin antibody in PBS Tween 20 was added to all wells. Plates were incubated at 37°C for 1 hr (for peroxidase conjugated Sheep IGG Anti-rat albumin antibodies, Cappel #55776 diluted 1:500 from 36.6 mg/ml) and again washed 3X with PBS Tween 20. 0.25 mg/ml of ABTS substrate (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) for peroxidase (Sigma #A9941) was added to a citrate substrate buffer (pH 5.0) with 0.01% hydrogen peroxide and then added to antibody plates for color development for 40 min at room temperature in the dark. The peroxidase reaction was stopped with 0.32 % sodium fluoride solution and absorbance was read at 405 nm using a BMG Optima plate reader.

Control conditions as discussed in the Examples below are defined as follows:

1) HS+MG= BD Hepato-STIM™ media on Matrigel;

2) HS+TCPS= BD Heptostim media on tissue culture polystyrene;

3) Block TCPS: Block Media on tissue culture polystyrene (Examples 1 and 3 only)

Block media is the media formulation described in the journal article by Block GD, Locker J, Bowen WC, Petersen BE, Katyal S, Strom SC, Riley T, Howard TA, Michalopoulos GK, Population expansion, clonal growth, and specific differentiation patterns in primary cultures of hepatocytes induced by HGF/SF, EGF and TGF alpha in a chemically defined (HGM) medium, *J Cell Biol.* 1996 Mar;132(6):1133-49, and in U.S. Patent No. 6,043,092.

EXAMPLE 1

CYP 1A1/1A2 activity of the three ECM compositions was assessed using 7-ethoxyresorufin for human primary hepatocytes after 7 days in culture, as described above. Figure 1 illustrates the results of the assessment. The CYP activity of the three ECM protein compositions is comparable to or better than cells placed on standard tissue culture polystyrene, with collagen I + poly-L-orthinine showing the highest level of activity. Because functional activity is typically lost within three days of culture, CYP activity on day 7 indicates maintenance of cell function.

EXAMPLE 2

CYP 1A1/1A2 activity of the three ECM compositions was assessed using 7-ethoxyresorufin for rat primary hepatocytes on day 6, using the methods described above. Figure 2 illustrates the results of the assessment. The total CYP fluorescence was lower than most hits in Figure 1. Again, CYP activity for the three ECM compositions is consistently higher then baseline fluorescence, either HA alone or 7-ethoxyresorufin alone. The control wells in the figure are HS+Matrigel and HS+TCPS.

EXAMPLE 3

Levels of albumin secretion of human primary hepatocytes were obtained on day 7 using the assay described above. Figure 3 illustrates this data for the three ECM protein compositions. Data shows that albumin secretion is maintained in wells having the ECM protein composition, and that their albumin levels are comparable to control wells of tissue culture polystyrene. Because functional activity is typically lost within three days of culture, albumin activity on day 7 indicates the maintenance of cell function. This data is also indicates maintenance of CYP activity.

EXAMPLE 4

Levels of albumin secretion of rat primary hepatocytes for the three ECM protein compositions were obtained on day 6 as described above. Figure 4 illustrates this data. Again, the levels of activity of the ECM compositions are comparable or superior to the controls, indicating maintenance of albumin secretion, and therefore cell function.

EXAMPLE 5

A morphology study was performed on primary hepatocytes comparing the activity of Collagen I alone, Poly-L-ornithine alone, and Collagen I with Poly-L-ornithine. Figures 5A-5C show the morphology of the cells at day 4. 5A shows Collagen I alone, 5B, poly-L-ornithine alone, and 5C, collagen I + poly-L-ornithine. Cells cultured on collagen I alone (5A) are spread out, and cells cultured on poly-L-ornithine (5B)alone do not spread or survive. However, combining collagen I with poly-L-ornithine (5C) causes formation of multi-cellular aggregates that maintain liver function, as shown by biochemical results (CYP and albumin, Figures 1-4) and morphology, as much as hepatocytes that aggregate on BD Matrigel™ The data shows that the ECM composition is superior the individual ECM and active factor components alone.

All references and patents cited herein are hereby incorporated by reference.